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Chromosomal instability predicts metastatic disease in patients with insulinomas

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Abstract

Endocrine pancreatic tumors (EPTs) comprise a highly heterogeneous group of tumors with different clinical behavior and genetic makeup. Insulinomas represent the predominant syndromic subtype of EPTs. The metastatic potential of insulinomas can frequently not be predicted using histopathological criteria, and also molecular markers indicating malignant progression are unreliable because of the small number of cases per subtype studied so far. For the identification of reliable indicators of metastatic disease, we investigated 62 sporadic insulinomas (44 benign and 18 tumors with metastases) by means of comparative genomic hybridization (CGH). In addition, the role of *MEN1* (multiple endocrine neoplasia type 1) gene mutations was determined to assess specific chromosomal alterations associated with dysfunction of this endocrine tumor-related tumor suppressor gene. Only one case with a somatic *MEN1* mutation was identified (1527del7bp), indicating that the *MEN1* gene plays a minor pathogenic role in sporadic insulinomas. CGH analysis revealed that the total number of aberrations per tumor differs strongly between the benign and the malignant group (4.2 vs 14.1; $P < 0.0001$). Furthermore, chromosome 9q gain was found to be the most frequent aberration in both benign and malignant insulinomas, whereas chromosome 6q losses and 12q, 14q and 17pq gains are strongly associated with metastatic disease. Our study shows that chromosomal instability, as defined by ≥ 5 gains together with ≥ 5 losses, or total number of gains and losses ≥ 8 , rather than parameters such as tumor size and proliferation index, is the most powerful indicator for the development of metastatic disease in patients with sporadic insulinoma.

Endocrine-Related Cancer (2005) 12 435–447

Introduction

Endocrine pancreatic tumors (EPTs) represent 1–2% of all pancreatic neoplasms and are separated on the basis of their clinical manifestation into functioning (syndrome-related) and non-functioning tumors. Insulinomas are the most frequently detected functioning EPTs. They show evidence of β -cell differentiation and

clinical symptoms of hypoglycemia due to uncontrolled insulin production. Strategies for insulinoma treatment include surgical resection and, in the case of palliation, chemotherapy and anti-hypoglycemic medication (Chun & Doherty 2001, Komminoth *et al.* 2004). The only feature that separates benign from malignant disease is organ and/or lymph node infiltration or distant metastases (Komminoth *et al.* 2004).

When only a primary lesion is identified, however, no reliable markers for malignancy are available. At best a tumor diameter ≥ 2 cm, an increased mitotic index and necrosis seem to indicate an increased risk for malignancy (Hochwald *et al.* 2002). It is obvious that new indicators that reliably determine prognosis of malignancy are urgently needed.

Little is known about the molecular pathogenesis of insulinomas (Leothela *et al.* 2003, Moore *et al.* 2003). So far, only limited numbers of predominantly benign insulinomas have been genetically analyzed, indicating the involvement of the *MEN1* (multiple endocrine neoplasia type 1) gene at 11q13, and gain of chromosome 9q in early tumorigenesis (Zhuang *et al.* 1997, Görtz *et al.* 1999, Cupisti *et al.* 2000, Speel *et al.* 2001). *MEN1* is the susceptibility gene of the autosomal dominant familial *MEN1* cancer syndrome (Schussheim *et al.* 2001). Patients with an inherited mutation in this gene develop tumors in endocrine organs, including insulinomas in 10–35% of cases. Evidence for a direct involvement of the *MEN1* tumor suppressor gene in early insulinoma development was provided by a transgenic mouse model with a hemizygous deletion of the *MEN1* gene in pancreatic β -cells. Somatic deletion of the second allele resulted in insulinoma formation with multiple progression features (Bertolino *et al.* 2003). However, a role for the *MEN1* gene in human sporadic insulinomas is unclear. Its mutation rate seems to be rather low, ranging from 0 to 17% in the different studies described so far (Zhuang *et al.* 1997, Görtz *et al.* 1999, Cupisti *et al.* 2000, and references therein). On the other hand, we have recently shown that in a small series of predominantly benign insulinomas, gain of chromosome 9q is the most common alteration identified by comparative genomic hybridization (CGH) in 50% of insulinomas with a diameter ≤ 2 cm, with the smallest region of gain being 9q34 (Speel *et al.* 2001).

Chromosomal markers with high potential to discriminate malignant from benign insulinomas have not been described so far. DNA cytometry data suggest that DNA ploidy is unlikely to provide useful prognostic information for patients with insulinomas, because large ploidy changes are seldomly detected (Graeme-Cook *et al.* 1990, Böttger *et al.* 1997). The few malignant insulinomas that have been genetically analyzed by molecular allelotyping and CGH were mainly part of larger studies on EPTs, including different functioning and non-functioning subtypes (Chung *et al.* 1998, Speel *et al.* 1999, 2001, Stumpf *et al.* 2000, Zhao *et al.* 2001). Several markers for malignant or metastatic progression in EPTs have been indicated, in particular loss of chromosomes 1 (Ebrahimi *et al.*

1999), 3p (Chung *et al.* 1997, Hessman *et al.* 1999, Barghorn *et al.* 2001a), 3q (Chung *et al.* 1998, Guo *et al.* 2002), 6q22-24 (Barghorn *et al.* 2001b), 11q13 (Hessman *et al.* 1999), 17p (Beghelli *et al.* 1998), 22q (Chung *et al.* 1998, Wild *et al.* 2002) and X (Missiaglia *et al.* 2002). These do, however, require further validation in larger series of individual EPT subtypes, including insulinomas.

The objective of the present study was to determine by CGH the occurrence of DNA copy number losses and gains along all chromosome arms in benign and malignant insulinomas in order to assess the chromosomal markers with the highest potential to predict metastatic disease. These markers will be compared with the best indicators of increased risk for malignancy suggested by the WHO, i.e. tumor size and proliferation index. For this purpose we have extended our previously analyzed collection of mainly benign insulinomas (Speel *et al.* 2001) to a large group of 62 tumors, including 18 malignant cases. In addition, these tumors were examined for *MEN1* mutations to assess the impact of this gene in insulinomas, and a possible association of such mutations with particular CGH profiles.

Materials and methods

Tumor material and patient data

Sixty-two insulinomas (32 females, mean age 52.1 ± 18.6 years and 30 males, mean age 53.1 ± 15.7 years) were selected from the archives of the Departments of Pathology of the University Hospital, Zurich, Switzerland and the University Medical Centers of Rotterdam, Utrecht and Nijmegen, The Netherlands. The study protocol was approved by the institutional ethical committee, and all of the patients gave informed consent. The samples included 59 frozen and three paraffin-embedded insulinomas, which were all sporadic tumors and not associated with the inherited *MEN1* syndrome. The tumors were classified according to the most recent WHO classification (Kommitho *et al.* 2004). All insulinomas had hyperinsulinism followed by a hypoglycemia syndrome. Forty-four of the patients had localized disease, defined by (i) the absence of extra-pancreatic spread of the tumor as evidenced by computed tomography, magnetic resonance imaging or ultrasound scanning, and (ii) a complete absence of hypoglycemia (even during 72 h fasting) after successful removal of the tumor by surgery. Moreover, during follow-up of at least 1 year, these cases showed neither evidence of recurrent hypoglycemia at follow-up, nor recurrence or metastases by

radiology. No post-operative adjuvant therapy was given to these patients. Eighteen patients had advanced disease, with tumor spread into the surrounding soft tissue, lymph nodes or liver.

DNA extraction

DNA extraction was performed as described before (Speel *et al.* 2001). Genomic DNA from frozen samples was isolated by homogenizing approximately 5 mm³ of each sample prior to proteinase K treatment and DNA purification using the QIAamp DNA mini kit (Qiagen). Genomic DNA from paraffin-embedded tumor material was isolated from 5–10 µm thick tissue sections by this procedure after deparaffination. DNA quality was checked with agarose gel electrophoresis and quantified by spectrophotometry.

CGH and digital image analysis

CGH was performed with DNA isolated from the 62 insulinoma samples as previously described (Speel *et al.* 2001). Briefly, 2 µg tumor DNA were labeled with Spectrum Green-dUTPs (Vysis, Downers Grove, IL, USA) by nick translation (BioNick labeling kit; Life Technologies). Spectrum Red-labeled normal and sex-matched reference DNA (Life Technologies) was used for co-hybridization. The hybridization mixture consisted of 800 ng Spectrum Green-labeled tumor DNA, 800 ng Spectrum-Red labeled reference DNA and 15 µg human Cot-1 DNA (Life Technologies) dissolved in 12 µl hybridization buffer (50% formamide, 2 × SSC, 10% dextran sulfate, pH 7.0). Hybridization was carried out for 3 days at 37 °C to denatured (5 min at 75 °C in 70% formamide/2 × SSC, pH 7.0) normal male human metaphase spreads (Vysis). Slides were washed twice at 45 °C for 5 min in 50% formamide/2 × SSC, pH 7.0, followed by 5 min in PBS at room temperature. The chromosomes were counterstained with 0.2 µg 4,6-diamidino-2-phenylindole per ml Vectashield (Vector) for identification.

Digital images were collected from at least ten metaphases using the Metasystems Image Pro System black and white CCD camera (Altusheim, Germany) mounted on top of a Leica DMRE fluorescence microscope, equipped with DAPI, Spectrum Green, and Spectrum Red filter sets. The software Metasystems ISIS 4.4.25 program was used to calculate average green-to-red ratio profiles for each chromosome. At least ten observations per autosome and five observations per sex chromosome were included in each analysis. Gains and losses of DNA sequences were defined as chromosomal regions where the mean

green-to-red fluorescence ratio was above 1.20 and below 0.80 respectively. Over-representations were considered amplifications when the fluorescence ratio values in a subregion of a chromosomal arm exceeded 1.5. In negative control hybridizations, the mean green-to-red ratio occasionally exceeded the fixed 1.2 cut-off level at the following chromosomal regions: 1p32-pter, 16p, 19 and 22. Gains of these G-C-rich regions were therefore excluded from all analyses.

Statistical analysis of CGH results

The relationship between genomic alterations and malignancy was analyzed by using the chi-square test or the Fisher exact test, as appropriate. ANOVA was applied to compare the number of genomic alterations between benign and malignant insulinomas. A significance level of $P \leq 0.05$ was chosen.

Confirmation of CGH data by fluorescence *in situ* hybridization (FISH) analysis

To validate CGH data independently, touch preparations of 15 insulinomas were subjected to FISH as described previously (Görtz *et al.* 1999, Speel *et al.* 2001). The following probe combinations were applied: (i) centromere 9 (pMR9α) and a cosmid probe (c-ABL-8) containing the *cAbl* gene on chromosome 9q34, (ii) centromere 6 (p308) and a PAC probe (66H14) mapping to the 6q21 region, and (iii) centromere 11 (pLC11A) and a cosmid probe (c10B11) containing the *MEN1* gene at 11q13. Digoxigenin-labeled probes were detected by sheep anti-digoxigenin fluorescein- (Roche) and biotin-labeled probes by two layers of avidin-rhodamine connected by a biotinylated goat anti-avidin antibody (Vector). Probe visualization and nuclear counterstaining were carried out as described for CGH, and signal scoring and evaluation was performed as described previously (Görtz *et al.* 1999, Barghorn *et al.* 2001a).

MEN1 mutation analysis

MEN1 is a 9 kb gene which encompasses ten exons (first exon untranscribed). The remaining nine coding exons were examined in 43 insulinomas, 31 benign and 12 malignant tumors, by denaturing gradient gel electrophoresis (DGGE) and single-strand confirmation polymorphism (SSCP), as previously used in several studies (see Table 1) (Görtz *et al.* 1999, Perren *et al.* 2002). PCR amplifications were carried out using a programmable thermal cycler (DNA thermal cycler 9600; Perkin Elmer, Norwalk, CT, USA). After initial denaturation at 94 °C for 5–10 min, the DGGE PCR

Table 1 Primers used for *MEN1* mutation analysis (exons 2–10): modified from a previous study (Görtz et al. 1999)

Primer	Sequence	Length	Technique	Annealing temperature (°C)
M2cnewF	5'-CCCGCTTCACCGCCCAGAT-3'	272	DGGE	55
M2cnewR	5'-TGGAGGGTTTTGAAGAAGTGGGTCA-3'			
M2newF	5'-CCTTAGCGGACCCCTGGGA-3'	365	SSCP	58
M2newR	5'-ATAGAGGGCGCGCATGATAGA-3'			
M2NNF	5'-TCAACCGCGTCATCCCTACC-3'	405	SSCP	62
M2NR	5'-CACCTGCCGAACCTCACAAG-3'			
M3F	5'-GCACAGAGGACCCTCTTTCAT-3'	335	DGGE	55
M3R	5'-CTACAGTATGAAGGGGACAAGG-3'			
M4F	5'-TGGGCCATCATGAGACATAA-3'	272	DGGE	55
M4R	5'-CCCACAGCAAGTCAAGTCTG-3'			
M5F	5'-CCTGTTCCGTGGCTCATAAC-3'	148	DGGE	55
M5R	5'-CCTGGCCACTTCCCTCTA-3'			
M6F	5'-GGTGGCAGCCTGAATTATGA-3'	213	DGGE	55
M6R	5'-TTAGGGTCTCCCTTCTGCAC-3'			
M7F	5'-GGTGGGAGTGGAGATGGAGAGG-3'	398	DGGE	55
M7R	5'-GGACGAGGGTGGTTGGAAACTG-3'			
M8NF	5'-AGACCCCTTCAGACCCTACAGAG-3'	273	SSCP	67
M8R	5'-CCATCCCTAATCCGTACATGC-3'			
M9F	5'-CTGCTAAGGGGTGAGTAAGAGAC-3'	334	DGGE	55
M9R	5'-AAAAGTCTGACAAGCCCGTG-3'			
M10NF	5'-TTGCTCTCACCTTGCTCTCC-3'	409	SSCP	56
M10NNR	5'-CTTGATGGCGCTCGAGTT-3'			
M10a F	5'-CCTTGCTCTCACCTTGCTCT-3'	392	DGGE	55
M10aR	5'-TCTGGAAAGTGAGCACTGGA-3'			
M10b F	5'-GAGGGTCCAGTGCTCACTT-3'	423	DGGE	55
M10bR	5'-GGGTCTGAGCTGGAGAAAA-3'			

procedure comprises 40 cycles of denaturation at 94 °C for 1 min, annealing at 55–67 °C (see Table 1) for 1 min and extension at 72 °C for 1 min. Finally, PCR tubes were incubated at 72 °C for 10 min, at 98 °C for 8 min, at 55 °C for 30 min, at 37 °C for 30 min, and then at 0 °C. The SSCP PCR procedure used the following incubations: 35 cycles of denaturation at 94 °C for 75 s, annealing at 55–67 °C (Table 1) for 90 s and extension at 72 °C for 2 min. Finally, PCR tubes were incubated at 72 °C for 5 min and then at 4 °C. PCR amplifications were performed in 50 µl reactions with 1 ng genomic DNA, 1 × PCR buffer (Roche), 1.5 mM MgCl₂, 0.2 mM dNTPs, 50 pmol of each forward and reverse primer and 1 U AmpliTaq Gold (Roche).

For the DGGE analysis, 10% polyacrylamide gels with a 20–80% (in the case of exon 10a: 50–90%) denaturing gradient were used. A solution of 100% chemical denaturant consists of 7 M urea and 40% formamide. The gels polymerized by adding 1% ammonium persulfate and 0.05% tetramethylethylenediamine. Once the gradient gel has polymerized, a 10% polyacrylamide stacking gel is layered on top of this gradient gel; 10 µl PCR-amplified DNA was mixed with 3 µl PCR-amplified non-mutated DNA, denatured,

annealed, and loaded on the gel together with 5 µl Ficoll loading buffer. Electrophoresis was performed at 100 V for 15 h. After electrophoresis the DNA was visualized by silver staining as previously described (Görtz et al. 1999). Patient DNAs with an altered migration pattern on the DGGE gel were amplified again with primers without a GC-clamp, purified using the QIAquick PCR purification kit (Qiagen), and subjected to DNA cycle sequencing in sense and anti-sense direction using the TaqDyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Weiterstadt, Germany). This was followed by gel electrophoresis, data collection, and analysis on an automated DNA sequencer (Model 373A; Applied Biosystems).

For SSCP analysis, 10 µl denatured PCR products in stop buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) were loaded onto non-denaturing 6% polyacrylamide and 5% glycerol gels. Electrophoresis was carried out for 16 h at 8 W and 500–700 V at room temperature. After electrophoresis the DNA was visualized by silver staining. PCR products with an altered migration pattern on a SSCP gel were further purified and sequenced as described above for DGGE.

Ki67 immunohistochemistry

Ki67 antigen staining was performed on 4 µm thick paraffin-embedded tissue sections of 35 insulinomas (22 benign and 13 malignant) as described previously (Wessels *et al.* 2004). Briefly, sections were deparaffinized and treated with 10 mM citrate buffer (pH 6.0) in a microwave oven at 600 W for 15 min (antigen retrieval). Endogenous peroxidase was inactivated by treatment with 2% H₂O₂ in methanol, followed by incubation in 3% BSA/PBS to block non-specific binding of antibody conjugates. The sections were incubated for 30 min at 37 °C with the mouse monoclonal antibody MIB-1 directed against Ki67 (Dako) at a 1:100 dilution in 1% BSA/PBS, biotin-labeled horse anti-mouse antibody at a 1:200 dilution, and avidin-biotinylated peroxidase complex (Vector Laboratories, Burlingame, CA, USA) at a 1:50 dilution in 4×SSC. Peroxidase activity was visualized using diaminobenzidine/H₂O₂ (Sigma Chemical Co.) and sections were counterstained with hematoxylin and mounted in Entellan (Merck, Darmstadt, Germany). Positive and negative control tissues were included. Positive nuclei were scored in at least 500 cells per sample. The Ki67 labeling index was expressed as the percentage of total cells that were immunopositive.

Results

General CGH findings

Figure 1 summarizes the DNA copy number changes detected by CGH in the 62 insulinomas. Genomic aberrations were found in 33 of the 44 benign and all malignant insulinomas. The average number of chromosomal aberrations per tumor was 7.2 ± 7.0 (range 0–28). Chromosomal gains (mean 3.9) were more common than chromosomal losses (mean 2.8), and in two tumors amplifications were identified, i.e. at 9q34 (2×) and 4p16 (1×). The most frequently involved specific genomic changes (in >20% of insulinomas) included loss of Xq in female patients (smallest region of interest (SRI): Xq21) (34%) and Y in male patients (43%), and gains of chromosomes 5q, 7pq, 9q, 17 and 20q, with the highest frequency of gains for chromosome 9q (58%). Interestingly, gain of chromosome 9q was detected at approximately the same frequency in both benign and malignant tumors (57 vs 61% respectively) with 9q34 most commonly included. When these tumors are compared with those without 9q alterations, the total number of aberrations and gains per tumor were significantly higher in the former group (9.1 ± 6.2 vs 4.6 ± 7.3 , $P=0.001$; and 5.5 ± 4.3 vs

1.7 ± 3.1 , $P<0.0001$ respectively), with gains of 7pq, 9p and 20q and loss of Xpq more frequently detected.

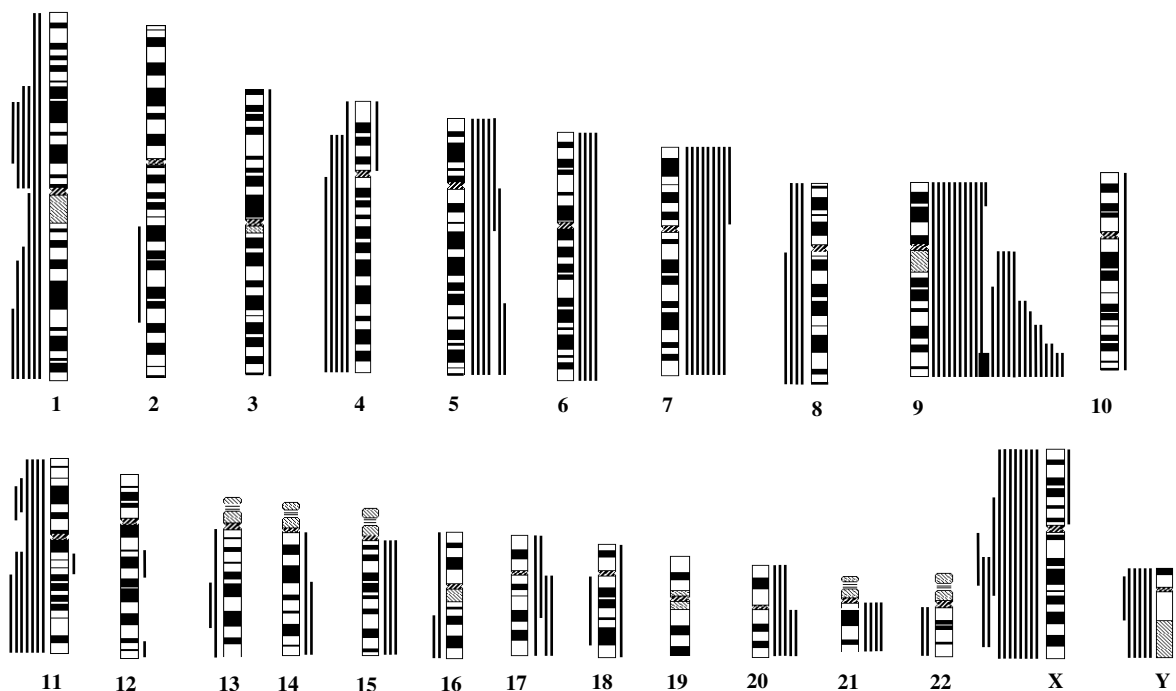
The obtained CGH results were validated in 15 insulinomas by FISH using chromosome 6-, 9- and 11-specific probe sets (Table 2 and Fig. 2). In general, the FISH results confirmed our CGH findings. Tumors without CGH alterations exhibited two or sometimes three copies of chromosome targets, indicating a diploid and triploid DNA content respectively (patients 1–7, 11–13), or showed alterations in small subpopulations (~20%) of cells (e.g. 11q13 loss in patients 2, 14 and 15). In the case of 9q alterations, always ≥ 3 copies of the 9q34-specific region were observed including two cases with an amplification (patients 10 and 15). Four cases showed tumor subpopulations with aberrant copy numbers of chromosome 9 targets indicating genetic heterogeneity (patients 10–12, 15).

CGH findings, tumor size and proliferation index in relation to metastatic disease

The total number of chromosomal changes, gains and losses all are strongly associated with malignancy ($P<0.0001$; Table 3A). In addition, marked differences in chromosome-specific alterations were observed between benign and malignant tumors (Fig. 1 and Table 3A). Particularly, losses of 2q, 3q, 6pq and 10q and gains of 4q, 7q, 12pq, 14q, 15q, 17pq, and 20pq were detected significantly more frequent in malignant insulinomas, with 6q loss (SRI: 6q14 and 21–22) (0% in benign vs 67% in malignant insulinomas), 12q gain (SRI: 12q24) (2% vs 50%), 14q gain (5% vs 50%) and 17pq gain (5 and 9% vs 56 and 61% respectively) correlating most evidently (in all cases $P<0.0001$). If the tumors are grouped according to the presence of 9q gains, the best common markers for malignancy are 6q loss and 7q, 14q and 17q gains (Table 3B). In addition, a number of genomic changes were exclusively identified in one or the other insulinoma group. Interestingly, the group with 9q gain shows gains and the group without 9q gain predominantly losses as parameters associated with malignancy (Table 3B).

Apart from differences in genomic alterations, benign insulinomas were found to be significantly smaller than malignant ones (diameter = 1.5 vs 3.8 cm respectively, $P<0.0001$). Because a diameter of 2 cm is an important parameter in insulinoma classification, we compared tumors with a size <2 cm with tumors ≥ 2 cm in diameter. This comparison revealed that an increase in the total number of aberrations, gains and losses was significantly correlated with a size ≥ 2 cm (Table 3A). Loss of 3q, 6pq and 22q and gains of 5pq,

Benign insulinomas (n=44)



Malignant insulinomas (n=18)

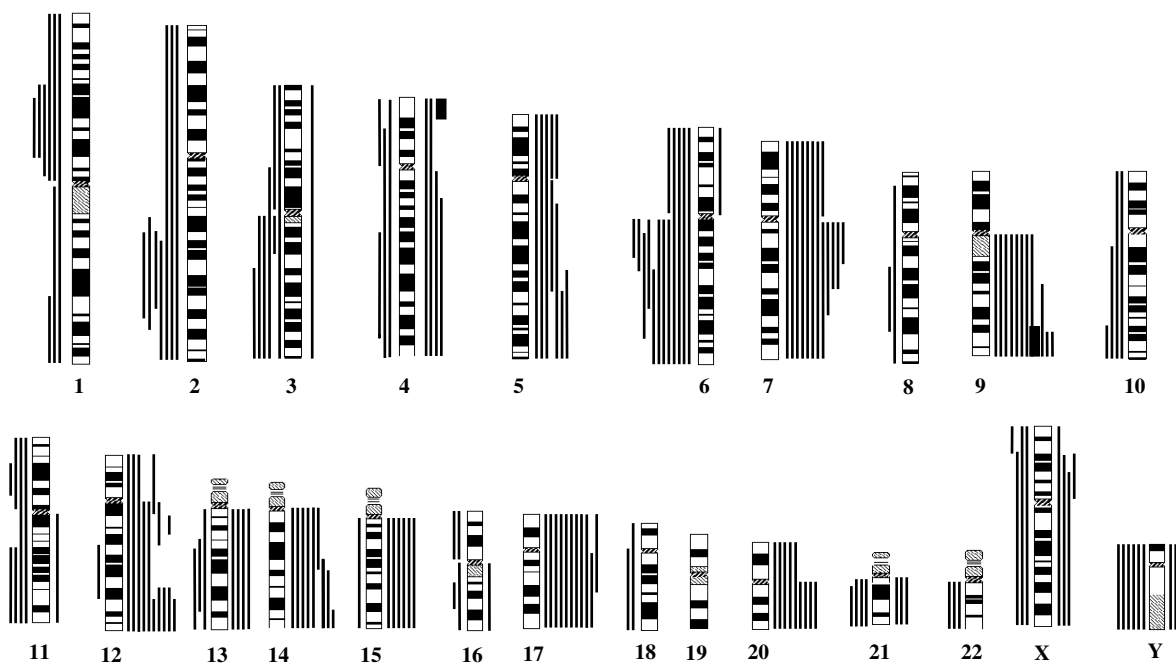


Figure 1 Summary of all DNA copy number changes detected by CGH in 44 benign and 18 malignant insulinomas. Vertical lines on the left of the chromosome ideograms indicate the number of cases showing losses of the corresponding chromosomal regions; vertical lines on the right indicate the number of cases showing gains. The bold lines represent amplifications at 4p16 and 9q34. Gains on 1p32-pter, 16p, 19, and 22 were not analyzed (see text).

Table 2 Double-target FISH results in 14 benign (cases 1–14) and one malignant (case 15) insulinomas

Tumor	CGH chr 6	6C:6q21 ^a	CGH chr 9	9C:9q34 ^a	CGH chr 11	11C:11q13 ^a
1	0	2:2	0	2:2	11pq [−]	1:1
2	0	2:2	0	2:2	0	2:1 (26%)
3	0	2:2	0	2:2	0	2:2
4	0	2:2	0	2:2	0	2:2
5	0	2:2	0	2:2(41%), 3:3 (30%)	0	2:2
6	0	2:2	0	2:2, 2: >2 (27%)	0	2:2
7	0	2:2	0	2:2	0	2:2
8	0	2:2	9qrg ⁺	2:3	0	2:2
9	0	2:2	9q34 ⁺	3:3	0	2:2
10	0	2:2	9pq ⁺	Area 1:3:3	11q13 ⁺	2:3
			9q34 ⁺⁺	Area 2:3:5, 3:>5 (Amp)		
11	0	3:3	9q34 ⁺	3:3 (37%), 4:4 (37%)	0	2:2
12	0	3:3	9q32–34 ⁺	3:3 (42%), 3:4 (24%)	11pq [−]	2:2
13	0	3:3	9q34 ⁺	3:6	0	3:3
14	6pq ⁺	3:3	0	2:2	0	2:1 (20%)
15	6q [−]	2:1 (34%)	9q ⁺	4:4 (44%), 4:8 (25%)	0	2:1 (22%)
			9q34 ⁺⁺	2:12 (5%; Amp)		

^aFISH results: 2–1 (n%), 2 copies of the centromere probe and 1 copy of the locus-specific probe in n% of nuclei (in the case of subpopulation of cells); Amp, amplification.

7q, 12q, 14q, 17pq and 21q were also significantly correlated with a tumor size ≥ 2 cm (Table 3A).

Another indicator of risk of malignancy is a proliferation index of $\geq 2\%$ measured by immunohistochemistry of Ki67 (MIB-1 monoclonal antibody). Comparison of 22 benign and 13 malignant insulinomas revealed only one benign and four malignant insulinomas showing a proliferation index $\geq 2\%$. This finding did not reach statistical significance when comparing benign and malignant tumors. Gain of chromosome 12q was the only parameter significantly correlated with a proliferative rate of $\geq 2\%$ (60 vs 13% in the group with a proliferation index $< 2\%$ respectively, $P=0.044$).

Table 4 shows these parameters as markers in univariate and bivariate analysis with the percentages of correctly classified tumors (sensitivity and specificity) and their odds ratios. The best classification markers for malignant behavior according to sensitivity in univariate analysis are the total number of aberrations (≥ 8), gains (≥ 5) and the size of the tumor (≥ 2 cm in diameter). A combination of the total number of gains (≥ 5) and losses (≥ 5) provides an even better sensitivity as shown by bivariate analysis with two independent markers.

MEN1 mutation analysis

MEN1 mutation analysis was performed on 43 sporadic insulinomas from which sufficient DNA was available. Only one insulinoma (male, 42 years old) showed a somatic 7 bp deletion in exon 10 (position

1527) of the *MEN1* gene, as shown in Fig. 2D–F. CGH analysis of this benign tumor showed gains of 5pq, 6pq and 21q. Although loss of chromosome 11q was not identified by CGH, FISH analysis revealed loss of the *MEN1* gene in 20% of cells (see above and Table 2, patient 14) suggesting deletion of the wildtype locus. A known polymorphism in exon 2 (S145S) was found in an additional tumor.

Discussion

We have performed a comprehensive genome-wide survey of DNA copy number changes in a series of 62 human sporadic insulinomas with the goal to identify genetic alterations that discriminate benign tumors from cases with metastatic progression. Particularly the CGH detection of a high number of confined chromosomal alterations per tumor was found to strongly correlate with metastatic disease in this EPT subtype. In addition, this study provides further evidence that gain and/or amplification of chromosome 9q, rather than *MEN1* mutations, is a frequent event in sporadic insulinomas that may be of pathogenetic importance.

Our data strongly indicate that insulinoma progression is driven by the accumulation of genetic changes, as is also known to occur in other types of human carcinomas (Nowak *et al.* 2002, Draviam *et al.* 2004, Vogelstein & Kinzler 2004). A marked distinction between benign and malignant insulinomas can be made on the basis of the number of genomic

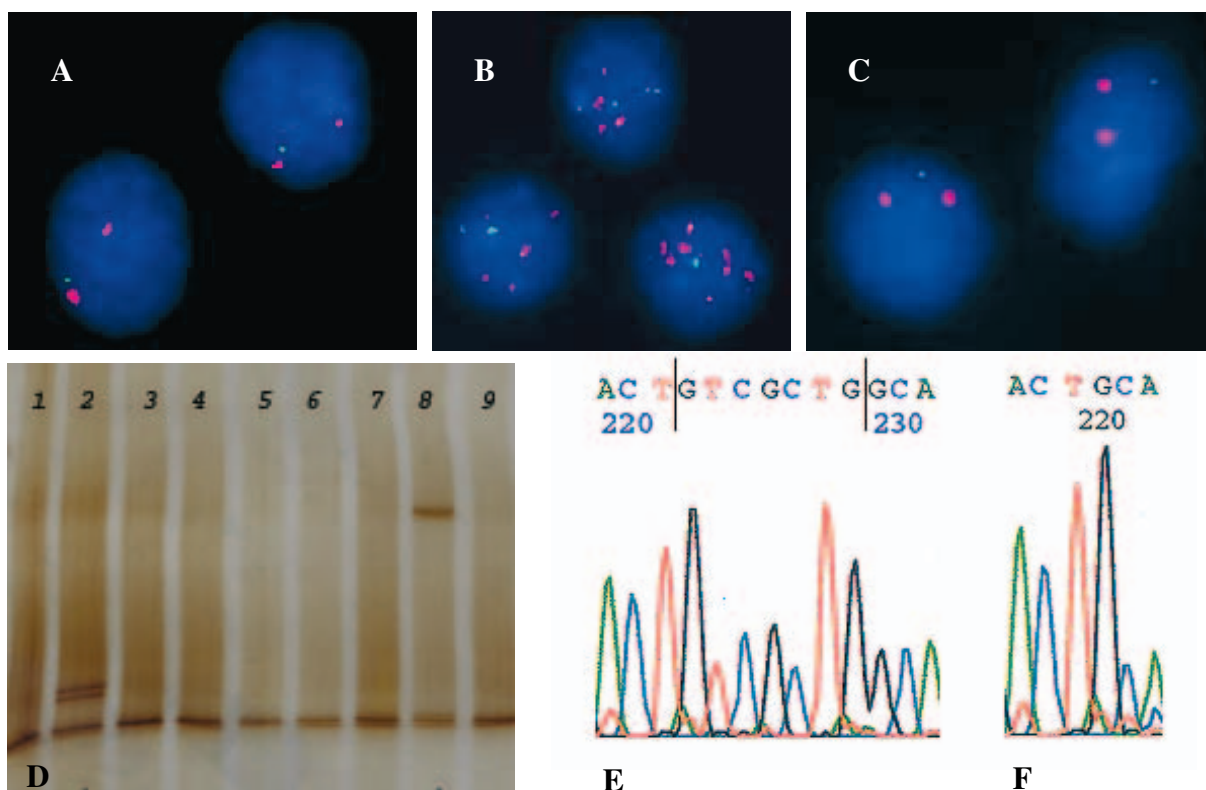


Figure 2 Results of representative examples of insulinoma cases examined by FISH (see Table 3) and MEN1 mutation analysis. (A) Loss of 6q21 (green; arrow) with respect to centromere 6 (red) in patient 15. (B) Gain and amplification of 9q34 (red) with respect to centromere 9 (green; arrow) in patient 10. (C) Loss of 11q13 (green; arrow) with respect to centromere 11 (red) in patient 2. (D) SSCP analysis of exon 10 of the *MEN1* gene in seven tumors with a negative control (lane 1) and a positive control (lane 2). Lane 8 represents DNA from patient 14 (Table 3) harboring a *MEN1* mutation. (E) The normal *MEN1* sequence profile of exon 10, with the part deleted in (F) between the vertical lines. (F) Sequence profile of patient 14 with the *MEN1* mutation, a 7bp deletion (GTCGCTG) in exon 10.

alterations per tumor as identified by CGH. The most prominent DNA copy number changes enabling this differentiation include loss of chromosome 6q (SRI 6q14 and 6q21-22) and gains of 7q, 12q (SRI 12q24), 14q and 17pq. In comparison with currently used indicators of malignancy, including tumor size >2cm and proliferative index $\geq 2\%$, high numbers of chromosomal alterations are significantly more powerful for this purpose (Table 4). The most potential indicators for metastatic progression detected in our study include: (i) a combination of gains and losses (both ≥ 5 per tumor), (ii) the total number of aberrations (≥ 8 per tumor), (iii) a combination of losses (≥ 5 per tumor) and 17q gain, and (iv) the total number of gains (≥ 5 per tumor). Thus, our data suggest that chromosomal instability (CIN), an abnormal cell state with elevated chromosomal gains and losses, is the optimal predictor for malignant progression in sporadic insulinomas. It will be interesting to identify the

target genes that might be involved in the development of CIN (Draviam *et al.* 2004).

The identified chromosomal regions altered in insulinomas point to loci containing putative oncogenes and tumor suppressor genes. The most frequent aberration identified by CGH in about 60% of both benign and malignant insulinomas is the increase of chromosome 9q copy numbers. This extends our previous findings in a small group of benign insulinomas and endorses the importance of this genetic event in early tumor development (Speel *et al.* 2001). Moreover, insulinomas with 9q gain harbored a significantly higher number of aberrations, especially gains, than the remaining tumors, and this difference in genetic makeup suggests the evolution along two different genetic pathways in insulinoma development. The SRI is located at 9q34 and this region was found amplified in two insulinomas. Gain and/or amplification of this region have been reported in other

Table 3 Parameters associated with malignancy and tumor size (A) or 9q gain (B)

A						
	Benign (<i>n</i> = 44)	Malignant (<i>n</i> = 18)	<i>P</i>	Size < 2 cm (<i>n</i> = 40)	Size ≥ 2 cm (<i>n</i> = 22)	<i>P</i>
Total aberrations	4.2 ± 4.6	14.1 ± 6.7	< 0.0001 ^a	4.7 ± 4.8	11.9 ± 8.0	< 0.0001 ^a
Losses	1.7 ± 2.5	5.7 ± 4.5	< 0.0001 ^a	1.7 ± 2.5	5.0 ± 4.5	< 0.0001 ^a
Gains	2.5 ± 3.3	7.3 ± 4.4	< 0.0001 ^a	2.8 ± 3.1	6.1 ± 5.2	< 0.0001 ^a
Mean size (cm)	1.5 ± 0.5	3.8 ± 2.3	< 0.0001 ^a	—	—	—
2q loss	5%	39%	0.002 ^c	8%	27%	NS ^b
3q loss	0%	33%	< 0.0001 ^c	3%	23%	0.010 ^b
4q gain	0%	22%	0.005 ^c	0%	18%	0.025 ^b
5p gain	11%	28%	NS ^c	8%	32%	0.033 ^b
5q gain	16%	39%	NS ^c	13%	41%	0.025 ^b
6p loss	0%	28%	0.001 ^c	0%	23%	0.008 ^b
6q loss	0%	67%	< 0.0001 ^c	5%	45%	< 0.0001 ^b
7q gain	18%	67%	0.001 ^c	20%	55%	0.012 ^b
10q loss	0%	22%	0.005 ^c	0%	18%	0.025 ^b
12p gain	0%	22%	0.005 ^c	0%	18%	0.025 ^b
12q gain	2%	50%	< 0.0001 ^c	8%	32%	0.033 ^b
14q gain	5%	50%	< 0.0001 ^c	5%	41%	0.001 ^b
15q gain	7%	33%	0.014 ^c	8%	27%	NS ^b
17p gain	5%	56%	< 0.0001 ^c	10%	36%	0.029 ^b
17q gain	9%	61%	< 0.0001 ^c	13%	45%	0.010 ^b
20p gain	7%	28%	0.039 ^c	8%	23%	NS ^b
20q gain	11%	50%	0.002 ^c	18%	32%	NS ^b
21q gain	9%	17%	NS ^c	3%	27%	0.011 ^b
22q loss	5%	22%	NS ^c	3%	23%	0.033 ^b

B							
	Insulinomas <i>with</i> 9q gain				Insulinomas <i>without</i> 9q gain		
Parameter	Benign (<i>n</i> = 25)	Malignant (<i>n</i> = 11)	<i>P</i>	Parameter	Benign (<i>n</i> = 19)	Malignant (<i>n</i> = 7)	<i>P</i>
Common parameters							
6q loss	0%	64%	< 0.0001 ^c	6q loss	0%	71%	< 0.0001 ^c
7q gain	32%	73%	0.034 ^c	7q gain	0%	57%	0.002 ^c
14q gain	8%	45%	0.018 ^c	14q gain	0%	57%	0.002 ^c
17q gain	16%	73%	0.002 ^c	17q gain	0%	43%	0.013 ^c
Group-specific parameters							
12p gain	0%	27%	0.023 ^c	1p loss	5%	57%	0.010 ^c
12q gain	4%	64%	< 0.0001 ^c	2q loss	0%	43%	0.013 ^c
13q gain	0%	27%	0.023 ^c	3q loss	0%	57%	0.002 ^c
17p gain	8%	73%	< 0.0001 ^c	6p loss	0%	57%	0.002 ^c
20q gain	20%	64%	0.020 ^c	21q loss	0%	43%	0.013 ^c
				22q loss	5%	57%	0.010 ^c
				Y loss	5%	57%	0.010 ^c
				5q gain	5%	57%	0.010 ^c

^aANOVA analysis.^bchi-square test.^cFisher exact test.

NS = not significant.

neoplasms, including neuroendocrine tumors (Dannen-berg *et al.* 2000, Figueiredo *et al.* 2000, Garcia *et al.* 2002), schwannomas (Warren *et al.* 2003), and entero-pathy-type T-cell lymphomas (Baumgärtner *et al.* 2003). There are several candidate genes localized in this region, i.e. the oncogene *VAV2* (Booden *et al.*

2002), *CDK9*, a co-regulator of the progression through the cell cycle (Napolitana *et al.* 2002), *Notch-1*, a transmembrane receptor promoting differentiation of T-cells (Aster *et al.* 2000), *LMX1B*, a LIM-homeodomain-containing protein involved in a variety of developmental events (Hobert & Westphall 2000),

Table 4 Parameters for assessing malignant behavior

Parameter	Specificity ^a	Sensitivity ^b	OR (95% CI)
Univariate analysis			
Total no. of aberrations ≥ 8	82	83	22.5 (5.2–96.6)
Total no. of gains ≥ 5	89	78	27.3 (6.4–116.3)
Total no. of losses ≥ 5	89	50	7.8 (2.1–29.0)
6q loss	100	67	>86 (9.4–785.2)
14q gain	95	50	21 (3.9–114.1)
17q gain	91	61	15.7 (3.9–63.6)
Size ≥ 2 cm	82	76	15.8 (4.1–60.7)
Proliferative index (Ki67) ≥ 2%	95	31	9.3 (0.9–95.6)
Bivariate analysis			
Total no. of gains ≥ 5, and total no. of losses ≥ 5	77	89	20.9 (4.5–96.0)
17q gain, and total no. of losses ≥ 5	80	83	15.8 (4.0–61.6)

OR = odds ratio, 95% CI = 95% confidence interval.

^aPercent of benign tumors without the parameter.

^bPercent of malignant insulinomas with the parameter.

TSC1, the tuberous sclerosis gene 1, and *cAbl*, playing a role in chronic myeloid leukemia (Goldman & Melo 2003). In TSC patients, insulinomas are occasionally detected. In these tumors, mutations in *TSC1* or *TSC2* (at 16p13.3) in combination with loss of the wildtype allele result in stimulation of cell growth and proliferation via dysregulation of mTOR activity (Davoren & Epstein 1992, Yong *et al.* 2004). In our series of insulinomas we noticed often ≥3 copies of 9q34 instead of loss. The intriguing finding that duplication/amplification of mutated alleles may also lead to tumorigenesis (Zhuang *et al.* 1998) should be examined in this respect. The oncogenic significance of the *cAbl* gene in insulinomas is unknown, although we identified two cases with amplification of this locus by FISH analysis, and *cAbl* overexpression has been reported in two rat insulinoma cell lines (DeAizpurua *et al.* 1997). Additional research is needed to clarify its possible role in the development of insulinomas.

Despite the importance of *MEN1* gene mutations in EPTs, we only identified one case with a *MEN1* mutation in a group of 43 insulinomas. In this tumor the wildtype allele was also lost. This finding is in agreement with other studies suggesting a minor role for *MEN1* in human insulinomas (Zhuang *et al.* 1997, Görtz *et al.* 1999, Cupisti *et al.* 2000, Gumbs *et al.* 2002, Moore *et al.* 2003). However, other mechanisms of gene inactivation may be involved in insulinoma development. Promoter hypermethylation of the *MEN1* gene has not been detected so far (Chan *et al.* 2003). The many different candidate proteins found to interact with menin may also implicate that other mechanisms can lead to down-regulation of the MEN1 protein (Chandrasekharappa & Teh 2003). This needs

to be further investigated using, for example, RT-PCR or immunohistochemistry (Cavallari *et al.* 2003). The high frequency of loss of heterozygosity in EPTs, including insulinomas, may also point to additional tumor suppressor gene loci at 11q (Chakrabarti *et al.* 1998, Speel *et al.* 2002).

Chromosome 6q loss proved to be the strongest chromosome-specific marker to classify the metastatic potential of insulinomas, because it was only detected in the malignant tumors. This is in agreement with a previous study, in which we mapped the SRI to 6q22-24 in sporadic EPTs in general (Barghorn *et al.* 2001b). Several candidate tumor suppressor genes are located in the common region of deletion, including *AIM1* (absent in melanoma 1), a beta-gamma-crystallin superfamily member inhibiting cellular growth by possible interactions with the cytoskeleton (Ray *et al.* 1997), *CCNC* (cyclin C) (Polly *et al.* 2000), *PTPRK* (receptor-type protein-tyrosine phosphatase kappa), possibly involved in the regulation of cell contact and adhesion via dephosphorylation of catenins or cadherins (Yang *et al.* 1996), *CX43* (connexin 43), a gap junction protein involved in intercellular communication (Mesnil 2002), and *LOT1* (*PLAGL1/ZAC1*). LOT1 is a zinc-finger nuclear transcription factor that regulates the type 1 receptor for pituitary adenylate cyclase-activating polypeptide, an important mediator of autocrine control of insulin secretion in the pancreatic islet. Furthermore, it possesses anti-proliferative effects and appears to be epigenetically silenced in different types of cancer, including parathyroid adenomas (Pagotto *et al.* 2000, Abdollahi *et al.* 2003). Future studies will determine if these genes are involved in insulinoma oncogenesis.

Deletions of other chromosomal regions that were reported to be associated with malignant behavior of EPTs, such as 1p, 3p, 3q, 11q, 17p, 22q and X (Chung *et al.* 1997, 1998, Beghelli *et al.* 1998, Ebrahimi *et al.* 1999, Hessman *et al.* 1999, Barghorn *et al.* 2001a,b, Guo *et al.* 2002, Missiaglia *et al.* 2002, Wild *et al.* 2002), were only occasionally observed in this CGH study on insulinomas. Losses of chromosome 3p and 3q were identified significantly more frequently in the malignant insulinomas, but only at low frequencies of 17 and 33% respectively. This holds also true for the association of 1p and 22q losses with malignancy in the group of insulinomas without 9q gain. These results indicate that the reported alterations are infrequent in insulinomas. However, it cannot be excluded that small deletions and gains are missed by CGH analysis due to the resolution of ~5–10 Mb. This might also underscore to some extent the frequency of alterations yet identified in this study. Gains of chromosomes 7pq, 12q, 14q and 17pq have been frequently reported in many types of cancer and are also commonly observed in sporadic gastrointestinal neuroendocrine tumors, including EPTs (Knuutila *et al.* 1998, Terris *et al.* 1998, Stumpf *et al.* 2000, Tönnies *et al.* 2001, Zhao *et al.* 2001). However, so far no convincing evidence has been presented of specific genes on these chromosomes playing a role in EPTs in general or insulinomas in particular (Speel *et al.* 1999, Goebel *et al.* 2002).

In summary, our data show that benign insulinomas can be efficiently distinguished from tumors with metastatic progression on the basis of the chromosomal profile of the tumors. Furthermore, because these alterations are recurrently detected by CGH, the identified chromosomal regions may harbor candidate cancer genes that are important in insulinoma pathogenesis. Whereas the presence of 9q gain and/or amplification appears to be an important genetic event in insulinoma development, the *MEN1* gene is unlikely to be involved in this process.

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